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HOST RESPONSE AND INFLAMMATION

Immunomodulatory Effects of *Lactobacillus plantarum* on Inflammatory Response Induced by *Klebsiella pneumoniae*

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ABSTRACT Some respiratory infections have been associated with dysbiosis of the intestinal microbiota. The underlying mechanism is incompletely understood, but cross talk between the intestinal microbiota and local immune cells could influence the immune response at distal mucosal sites. This has led to the concept of enhancing respiratory defenses by modulating the intestinal microbiota with exogenous supplementation of beneficial strains. In this study, we examined the effect of *Lactobacillus plantarum* CIRM653 on the inflammatory response induced by the pathogen *Klebsiella pneumoniae*. Oral administration of *L. plantarum* CIRM653 to mice subsequently infected by *K. pneumoniae* via the nasal route (i) reduced the pulmonary inflammation response, with decreased numbers of lung innate immune cells (macrophages and neutrophils) and cytokines (mouse keratinocyte-derived chemokine [KC], interleukin-6 [IL-6], and tumor necrosis factor alpha [TNF-α]) in the bronchoalveolar fluid, and (ii) induced an immunosuppressive Treg response in lungs. *In vitro* coincubation of *L. plantarum* CIRM653 and *K. pneumoniae* with human dendritic cells and peripheral blood mononuclear cells resulted in decreased Th1 (IL-12p70 and interferon gamma [IFN-γ]) and Th17 (IL-23 and IL-17) and increased Treg (IL-10) cytokine levels compared to those observed for *K. pneumoniae*-infected cells. Neither *K. pneumoniae* nor *L. plantarum* CIRM653 had any effect on cytokine production by intestinal epithelial cells *in vitro*, but the induction of the NF-κB pathway and IL-8 and IL-6 production by *K. pneumoniae* in airway epithelial cells was significantly reduced when the pathogen was coincubated with *L. plantarum* CIRM653. The remote IL-10-mediated modulation of the *K. pneumoniae* inflammatory response by *L. plantarum* CIRM653 supports the concept of immunomodulation by beneficial bacteria through the gut-lung axis.

KEYWORDS *Lactobacillus*, gut-lung axis, inflammation

Bacterial pneumonia is a potentially severe disease. It can contribute to sepsis and is difficult to eradicate when caused by multiantibiotic-resistant strains. The Gram-negative bacterium *Klebsiella pneumoniae* is a major cause of nosocomial infections, including pneumonia, and most clinical strains have multiple-antibiotic resistance (1). The recent emergence of *K. pneumoniae* strains resistant to carbapenem antibiotics has left few treatment options and is associated with high mortality rates (2). These infections are characterized by a deregulated lung immune response that leads to excessive inflammation, with high levels of proinflammatory cytokines ("cytokine storm") and an extreme accumulation of neutrophils, which results in acute lung inflammation/acute lung injury (3–7). The lungs are continuously exposed to environmental antigens and possess strong mechanisms of defense to protect against pathogens responsible for respiratory tract infections. Innate immune cells such as airway epithelial cells, alveolar

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macrophages, and dendritic cells (DCs) provide the first line of lung defense and coordinate adaptive immunity to eliminate pathogens. An emerging concept based on the gut-lung axis hypothesis suggests that activation of lung immunity is in part under the control of intestinal microbiota (8–13). Experiments performed *in vivo* with *K. pneumoniae* showed that dysbiosis in the composition of the intestinal microbiota is associated with modifications of the lung immune response and consequently with pathogenic outcomes in the respiratory tract (14–17). In addition, previous epidemiological studies showed that *K. pneumoniae* strains responsible for nosocomial infections originate from the gastrointestinal reservoir of the patients (18). Hence, oral administration of beneficial bacteria could be an alternative therapeutic strategy to prevent and/or treat lung infections induced by *K. pneumoniae*. Not only would these bacteria limit the intestinal proliferation of *K. pneumoniae*, they would also modulate the inflammatory response and thereby improve lung immunity.

We previously selected a beneficial strain, *Lactobacillus plantarum* CIRM653, on the basis of its ability to disrupt *K. pneumoniae* colonization in different *in vitro* and *in vivo* models (19). In the present study, we assessed the distal contribution of the oral administration of this strain to the pulmonary inflammatory response in a mouse model of *K. pneumoniae*-induced pneumonia. The antagonist immunomodulatory effects of *L. plantarum* CIRM653 and *K. pneumoniae* observed *in vivo* prompted us to perform *in vitro* assays with immune and epithelial cells to investigate the underlying mechanisms. Our results suggest that beneficial bacteria have a distal impact on pathogens via modulation of the host immune system.

RESULTS

Daily oral administration of *L. plantarum* CIRM653 prevents innate cell recruitment and cytokine production in the lungs of *K. pneumoniae*-infected mice. Intranasal inoculation of *K. pneumoniae* into C57BL/6 mice led to significant bacterial burden and immune cell infiltration in the lung tissue after 24 h of incubation (Fig. 1A and B), with significant weight loss compared to noninfected mice (2.5% $\pm$ 1.8% for *K. pneumoniae*-infected mice versus 2.4% $\pm$ 0.9% for noninfected mice; $P = 0.04$) (see Fig. S1 in the supplemental material). The immune response was characterized by an increase in total leukocytes with high numbers of macrophages and neutrophils (Fig. 1B). Daily oral treatment of *K. pneumoniae*-infected mice with *L. plantarum* CIRM653 for 7 days before nasal administration of the pathogen significantly reduced the bacterial load (Fig. 1A) and the number of macrophages and neutrophils in the lung tissue (Fig. 1B). The same variation in cell numbers was observed in the animals' bronchoalveolar fluid (Fig. 1C and D). Administration of *L. plantarum* alone had no effect on the basal number of leukocytes compared to that in untreated control mice (Fig. 1A to D). Concomitant determination of the cytokine concentrations in the bronchoalveolar fluid showed increased levels of proinflammatory cytokines mouse keratinocyte-derived chemokine (KC), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) in mice infected with *K. pneumoniae* compared to those of the control group (Fig. 1E). Similar results were observed in lung tissues (data not shown). Oral administration of *L. plantarum* CIRM653 alone did not affect cytokine expression compared to that in control mice but significantly reduced cytokine levels in *K. pneumoniae*-infected mice (Fig. 1E). The level of the phospho-NF-κB p65 protein was higher in the lungs of *K. pneumoniae*-infected mice than in those of the control group (Fig. 1F). *L. plantarum* CIRM653 inhibited phospho-NF-κB p65 in mice treated with both *K. pneumoniae* and *L. plantarum* CIRM653 (Fig. 1F).

An immunosuppressive T cell response is induced by *L. plantarum* CIRM653 in the lungs of *K. pneumoniae*-infected mice. After 72 h of incubation, the immune response induced by *K. pneumoniae* was characterized by an increase in *T-bet* mRNA expression (Fig. 2A) and no more detection of a bacterial burden in the lungs of all groups of mice. No significant increase was observed for *ROR-*γ*t*, *il10*, *foxp3*, and *il2* mRNA expression in *K. pneumoniae*-infected mice compared to noninfected mice (Fig. 2A). Daily oral treatment of *K. pneumoniae*-infected mice with *L. plantarum* CIRM653

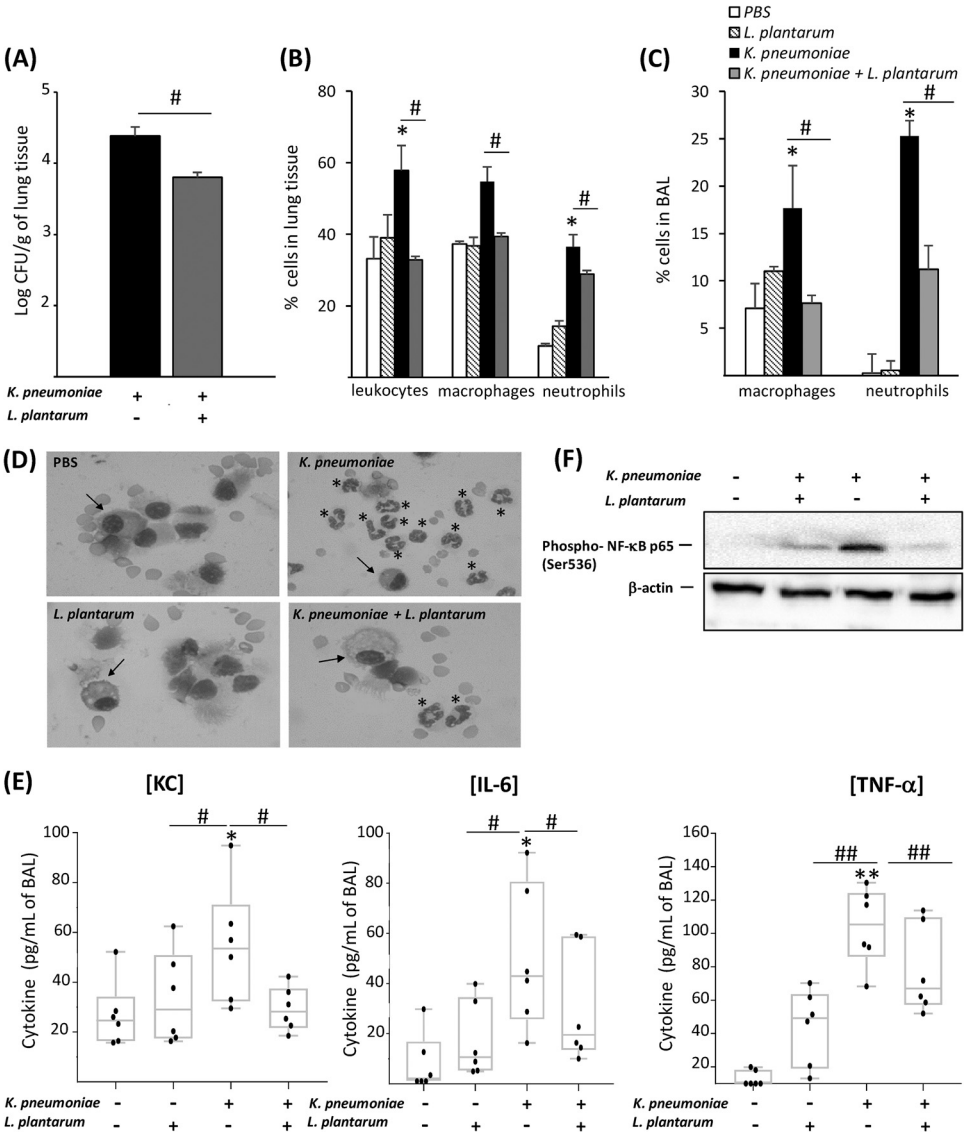

FIG 1 *L. plantarum* CIRM653 prevents innate responses in the lungs of *K. pneumoniae*-infected mice. Mice were inoculated daily by oral gavage with *L. plantarum* CIRM653 (100 μl containing 10^8 CFU) or PBS as a control for 7 days. On day 7, they were infected intranasally with 25 μl of a *K. pneumoniae* suspension (4.0×10^7 CFU/ml), and cell populations and cytokines were analyzed in the lungs 24 h after *K. pneumoniae* challenge. (A) Bacterial burden recovered from lungs of mice after *K. pneumoniae* infection. (B) Proportions (percent) of leukocytes, macrophages, and neutrophils in the lungs were analyzed by flow cytometry. (C) Proportions (percent) of macrophages and neutrophils related to total cells from BAL fluid. (D) Representative cytospin images showing macrophages (black arrows) and neutrophils (asterisks) from BAL fluid. (E) KC, IL-6, and TNF-α cytokine levels in BAL fluid from mice after *K. pneumoniae* infection were measured by an ELISA. (F) The presence of the phosphorylated NF-κB p65 (Ser536) and β-actin proteins in the lung tissues was detected by Western blotting. Each value represents the mean $\pm$ SEM for 6 to 8 mice. *, $P < 0.05$, and **, $P < 0.01$, compared with uninfected animals; #, $P < 0.05$, and ##, $P < 0.01$, compared with other groups.

significantly reduced *T-bet* and *il2* and induced *foxp3* and *il10* mRNA expression in the lung tissue (Fig. 2A). Administration of *L. plantarum* alone had no effect on the basal gene expression level compared to that in untreated control mice (Fig. 2A). Concomitant determination of the cytokine concentrations in lung tissues showed no significant difference in levels of the cytokines interferon gamma (IFN-γ), IL-23, and IL-10 in mice infected with *K. pneumoniae* and treated with *L. plantarum* CIRM653 compared to mice infected with *K. pneumoniae* alone (Fig. S2). In the mediastinal lymph nodes (MLNs), administration of *L. plantarum* CIRM653 increased the numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ cells compared to those in *K. pneumoniae*-infected mice (Fig. 2B).

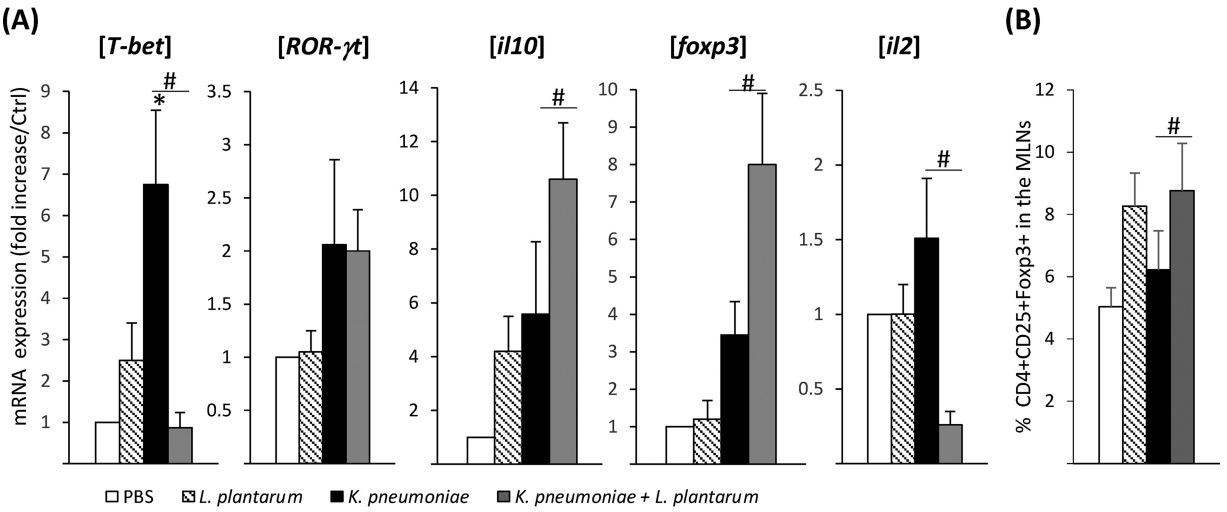

FIG 2 *L. plantarum* CIRM653 induces an immunosuppressive adaptive response in the lungs of *K. pneumoniae*-infected mice. Mice were inoculated daily by oral gavage with *L. plantarum* CIRM653 (100 μl containing 10^8 CFU) or PBS as a control (Ctrl) for 10 days. On day 7, they were infected intranasally with 25 μl of a *K. pneumoniae* suspension (4.0×10^7 CFU/ml), and cytokines and cell populations were analyzed in the lungs and the mediastinal lymph nodes (MLNs), respectively, 72 h after *K. pneumoniae* challenge. (A) Quantitative real-time PCR analysis for mRNA levels of *T-bet*, *ROR-γt*, *il10*, *foxp3*, and *il2* in lung tissues. (B) Proportions (percent) of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in the mediastinal lymph nodes were analyzed by flow cytometry. Each value represents the mean ± SEM for 6 to 8 mice. *, $P < 0.05$ compared with uninfected animals; #, $P < 0.05$ compared with other groups.

***L. plantarum* CIRM653 has no effect on dendritic cell maturation but induces IL-10 production in *K. pneumoniae*-infected dendritic cells.** Stimulation of DCs by *K. pneumoniae* induced a statistically significant increase in the percentage of CD86-positive cells and a decrease in the percentage of DC-SIGN-positive cells (Fig. 3A). No such effect was observed when DCs were incubated with *L. plantarum* CIRM653, while coincubation of *K. pneumoniae* with *L. plantarum* CIRM653 induced a profile similar to that observed with *K. pneumoniae* (Fig. 3A).

Determination of the concentrations of IL-12p70 and IL-23 secreted by dendritic cells showed an increase when the cells were incubated with *K. pneumoniae* compared to noninfected cells (Fig. 3B). Infection of DCs with both *K. pneumoniae* and *L. plantarum* CIRM653 gave rise to levels of these proinflammatory cytokines that were similar to those observed with *K. pneumoniae* alone, irrespective of the multiplicity of infection (MOI) of *L. plantarum* CIRM653 tested (Fig. 3B). Parallel determination of the amount of the anti-inflammatory cytokine IL-10 showed that DCs infected by *K. pneumoniae* alone induced no increase in its secretion, whereas coinfection with *L. plantarum* CIRM653 resulted in increased IL-10 production in a dose-dependent manner compared to noninfected DCs (Fig. 3B). No significant increase in IL-12p70, IL-23, and IL-10 was observed for dendritic cells stimulated with *L. plantarum* CIRM653 alone (Fig. 3B).

***L. plantarum* CIRM653 modulates proliferation and cytokine levels in PHA-stimulated PBMCs upon *K. pneumoniae* infection.** The proliferative response of phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMCs) increased in a dose-dependent manner following *K. pneumoniae* infection (Fig. 4A). Coinfection of these cells with *K. pneumoniae* and *L. plantarum* CIRM653 resulted in a significant decrease in lymphocyte proliferation ($P < 0.05$), with values comparable to those for PHA-stimulated control cells (Fig. 4A).

PHA-stimulated human PBMCs infected with *K. pneumoniae* secreted high levels of the proinflammatory cytokines IFN-γ and IL-17A (Fig. 4B), and coincubation with *L. plantarum* CIRM653 resulted in lower levels of these cytokines in the cell supernatants. Regarding the production of anti-inflammatory cytokines, the level of IL-10 also increased when incubation was performed with *K. pneumoniae* alone compared to that for the control cells but was lower than that observed with *L. plantarum* CIRM653 alone

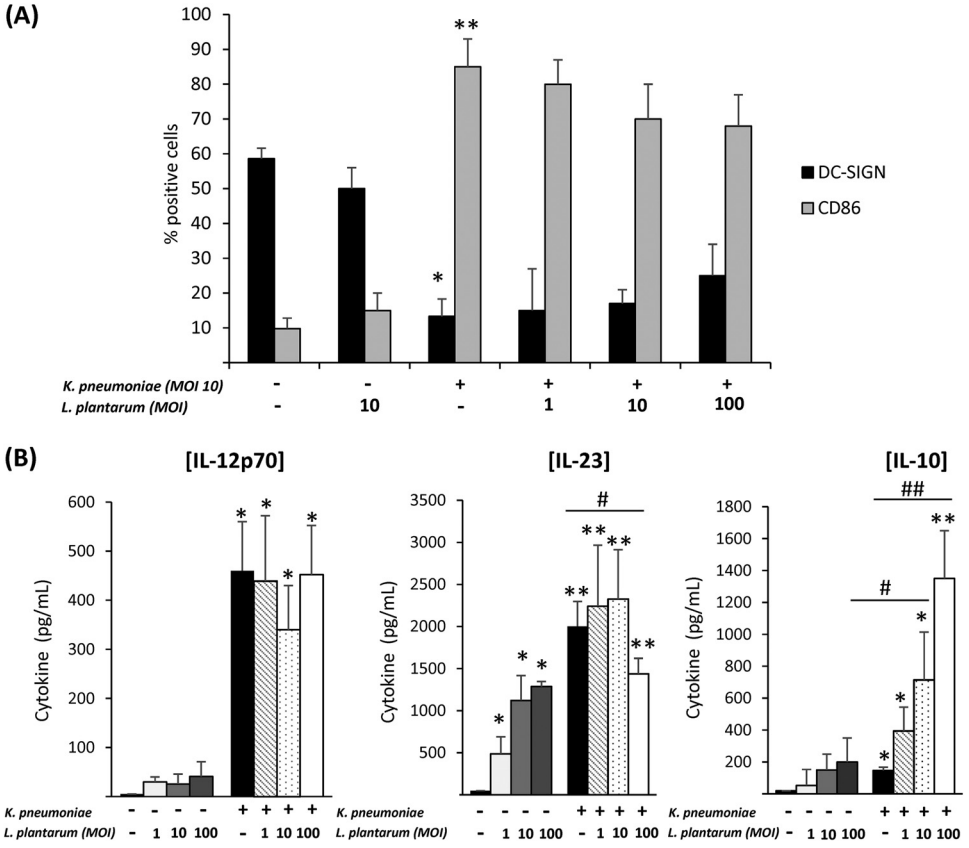

FIG 3 Effect of *L. plantarum* CIRM653 on response of *K. pneumoniae*-infected dendritic cells. Human monocyte-derived dendritic cells were exposed to UV-inactivated *K. pneumoniae* (MOI of 10) and UV-inactivated *L. plantarum* CIRM653 (MOI of 1 to 100) for 48 h. (A) The effects of DC functional maturation were determined by measuring the DC surface expression of DC-SIGN and CD86 by flow cytometry. (B) The secretion of the cytokines IL-12p70, IL-23, and IL-10 was measured by an ELISA. Values are the means ± SEM ($n = 3$ to 4). *, $P < 0.05$; **, $P < 0.01$ (compared with noninfected DCs). #, $P < 0.05$; #, $P < 0.01$ (compared with DCs infected with *K. pneumoniae*).

(MOI of 100) (Fig. 4B). Coinfection of the cells with both *K. pneumoniae* and *L. plantarum* CIRM653 resulted in significantly larger amounts of IL-10 in the cell supernatants at the highest MOI tested (MOI of 100) (Fig. 4B) and consequently significantly decreased the Th1/Treg cell ratio in cytokine production (Fig. 4C).

***L. plantarum* CIRM653 reduces the proinflammatory response induced by *K. pneumoniae* only in human airway epithelial cell lines.** Contact of *K. pneumoniae* with A549 pulmonary epithelial cells, unlike with uninfected cells, induced the expression of the genes *il8* and *il6* (Fig. 5A and B) and the production of the corresponding cytokines (Fig. 5C and D) in a dose-dependent manner. No such increase was observed when A549 cells were incubated with *L. plantarum* CIRM653 alone (17.7- ± 2.0- and 1.9- ± 0.9-fold increases compared to control cells for *il8* and *il6* expression, respectively, and 630.1 ± 37.3 and 153.1 ± 24.2 pg/ml for IL-8 and IL-6 production, respectively). No significant change in the production of TNF-α, IL-1β, and IL-10 was observed after incubation of the cells with either *K. pneumoniae* or *L. plantarum* CIRM653 (data not shown). Coinfection of A549 cells with *L. plantarum* CIRM653 and *K. pneumoniae* resulted in decreases in both the expression and production of proinflammatory cytokines in an *L. plantarum* CIRM653 MOI-dependent manner (Fig. 5A to D). No such effect was observed when cells were coinfected with *K. pneumoniae* and UV-killed *L. plantarum* CIRM653 (1,427.19 ± 44.59 pg/ml for *K. pneumoniae* alone at an MOI of 100 and 1,445.16 ± 54.8 pg/ml with UV-killed *L. plantarum* CIRM653 at an MOI of 100 for IL-8 production). Infection of epithelial intestinal Caco-2 cells with either *K. pneumoniae* or *L. plantarum* CIRM653 did not elicit the expression and secretion of IL-8 and IL-6 compared to uninfected cells (Table 1).

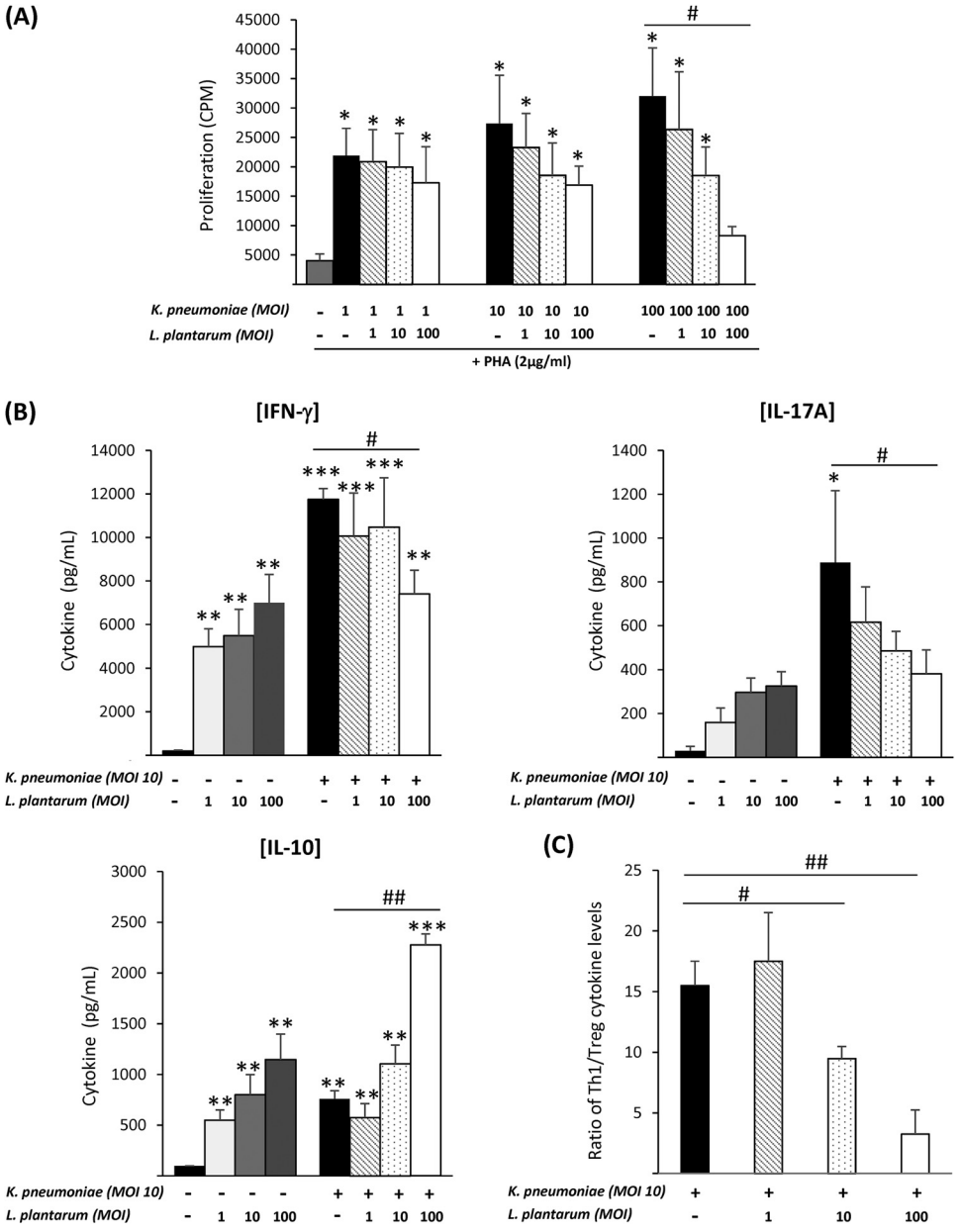

FIG 4 Anti-inflammatory effect of *L. plantarum* CIRM653 on PHA-stimulated lymphocytes upon *K. pneumoniae* infection. PBMCs were treated with PHA (2 μg/ml) and infected by UV-inactivated *K. pneumoniae* and *L. plantarum* CIRM653 at various concentrations (MOI of 1 to 100). (A) Proliferation was measured by [^{3}H]thymidine incorporation. (B) Supernatants were collected and analyzed for IFN-γ, IL-17A, and IL-10 by an ELISA. The results are representative of data from four experiments. (C) The Th1/Treg ratio was obtained by dividing the mean IFN-γ concentration by the mean IL-10 concentration. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$ (compared to stimulated/noninfected PBMCs). #, $P < 0.05$; ##, $P < 0.01$ (compared to PBMCs infected with *K. pneumoniae*).

Activation of the NF-κB pathway was marked in A549 cells infected with *K. pneumoniae* after 60 min (Fig. 5E). In contrast, the levels of p65 accumulation in cells infected with both *K. pneumoniae* and *L. plantarum* CIRM653 were significantly lower than those in cells infected with the pathogen alone ($P < 0.05$) (Fig. 5F).

In addition, infection of A549 epithelial cells with *K. pneumoniae* alone induced a statistically significant increase in IκB-α degradation compared to that observed when the cells were incubated with both *K. pneumoniae* and *L. plantarum* CIRM653 (Fig. 5E and F).

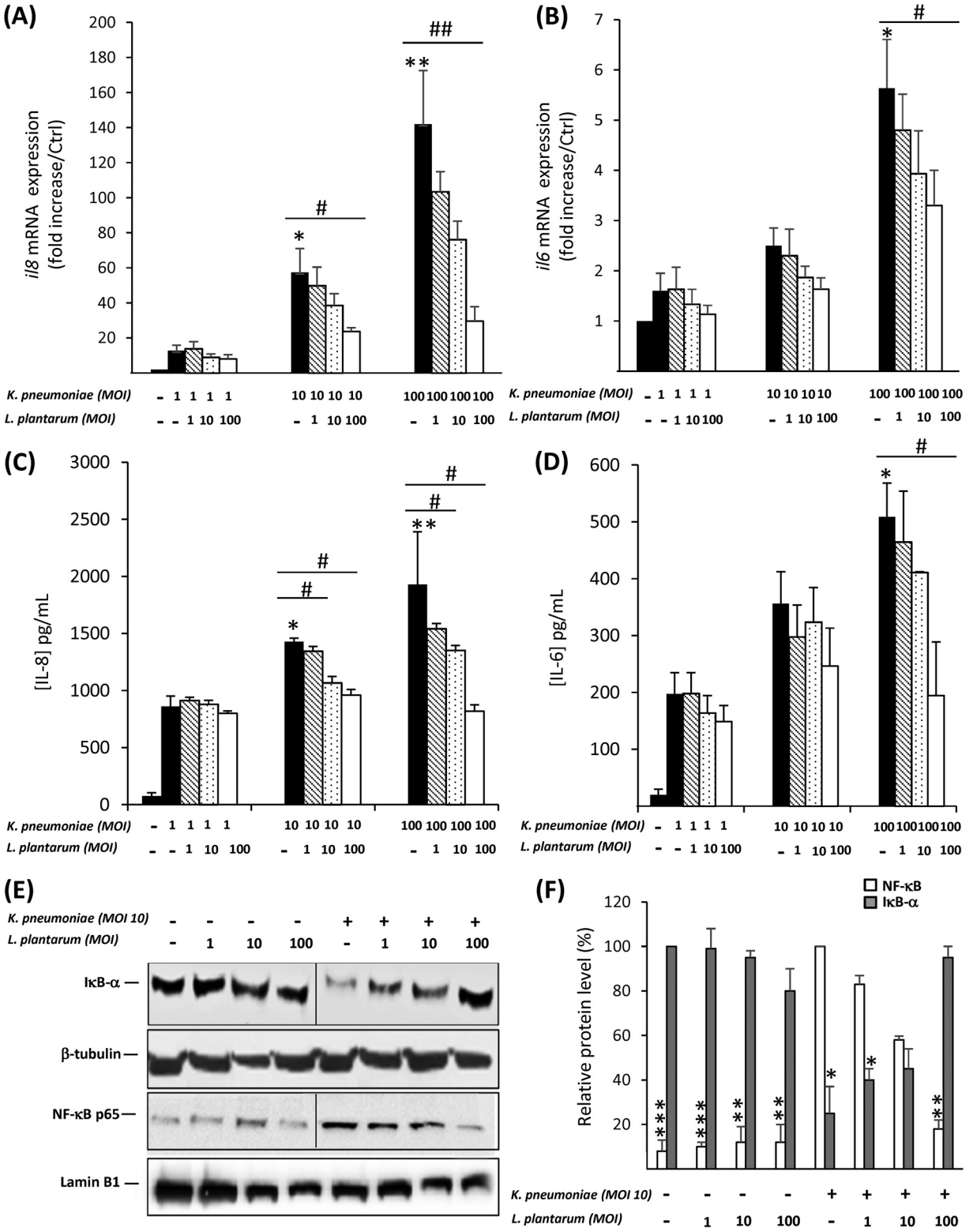

FIG 5 Effect of *L. plantarum* CIRM653 on inflammatory responses induced by *K. pneumoniae* in airway epithelial cells. A549 cells were infected with *K. pneumoniae* and *L. plantarum* CIRM653 for 3 h at different MOIs (MOIs of 1 to 100). (A and B) The levels of *il8* mRNA (A) and *il6* mRNA (B) were analyzed by real-time RT-PCR ($n = 6$). (C and D) IL-8 (C) and IL-6 (D) concentrations were analyzed by an ELISA. (E) The presence of the IκB-α and β-tubulin proteins in cytoplasmic extracts and p65 NF-κB and lamin B1 proteins in nuclear extracts was detected by Western blotting of A549 cells stimulated for 3 h with *K. pneumoniae* and/or *L. plantarum* CIRM653. Representative data are from three independent experiments, with gels spliced for labeling purposes (IκB-α and p65 NF-κB). (F) Densitometric analysis of the data in Fig. 4E by using Image Lab 2.0 software ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with noninfected A549 cells). #, $P < 0.05$; ##, $P < 0.01$ (compared with A549 cells infected with *K. pneumoniae*).

TABLE 1 Expression and production of proinflammatory cytokines in Caco-2 intestinal epithelial cells after incubation with *K. pneumoniae* or *L. plantarum* CIRM653[a]

Parameter	Value for:		
	Control	*K. pneumoniae*-infected cells	*L. plantarum*-infected cells
il8 mRNA	1	1.9 ± 0.2	1.6 ± 0.3
IL-8 concn (μM)	30.3 ± 70.4	57.4 ± 73.3	50.9 ± 59.1
il6 mRNA	ND	ND	ND
IL-6 concn (μM)	ND	ND	ND

[a]Caco-2 cells were infected with *K. pneumoniae* or *L. plantarum* CIRM653 (MOI of 100) for 3 h. Subsequently, *il8* and *il6* mRNA expression levels were analyzed by real-time PCR and are expressed as a fold increase compared to levels in control cells. Cells were washed and incubated in fresh medium containing antibiotics for 18 h. Cytokine concentrations were measured in the culture supernatants by an ELISA. Each value is the mean ± SEM of data from 3 to 8 independent experiments. ND, not detected.

DISCUSSION

Beneficial bacteria can modulate mucosal immune responses *in vitro* and *in vivo* by regulating innate immunity and the T cell response (20, 21). These emerging data gave rise to the concept of using intestinal nonpathogen microbiota species as candidates for the control of inflammatory responses at distal sites, including the lung. The mechanisms involved in this so-called "gut-lung axis" control are not clearly understood, but experiments have shown that intestinal bacteria (bacterial fragments or metabolites) enter the systemic circulation and modulate the lung immune response (22, 23). Immune cell migration from the gut to the lung could also play a distal role in the context of a common mucosal immune system (8, 9, 13, 24, 25). Various immunological mechanisms have been described for the local interaction between intestinal microbiota and mucosal immunity, including the control of both innate and adaptive immunity (14, 26–28). In this study, we examined the effect of *Lactobacillus plantarum* CIRM653 on the global (early and late) host immune response induced in lung tissue and cells by the pathogen *K. pneumoniae*. It is noteworthy that, in our experiments, *K. pneumoniae* had no effect on the inflammatory response in intestinal epithelial cell lines, unlike in respiratory epithelial cells, suggesting that intestinal cells "tolerate" the presence of *K. pneumoniae*. This is consistent with the facts that the human gastrointestinal tract is a reservoir for this pathogen and that this colonization process does not induce adverse intestinal disorders (29).

L. plantarum CIRM653 significantly reduced the innate immune response induced by the pathogen, with decreases in proinflammatory cytokine (KC, IL-6, and TNF-α) levels, both *in vivo* and *in vitro*, and *in vivo* infiltration of macrophages and neutrophils. Although most studies have shown an impact of beneficial bacteria on the innate immune response (30–32), a few recent reports have considered the possibility that these bacteria also produce an immunomodulatory effect at distal sites (17, 33–36). In our experiments, both *in vitro* and *in vivo*, the transcription factor NF-κB had to be activated to control the inflammatory response induced by *K. pneumoniae* in innate cells. Like many immune cells, innate cells, including epithelial cells, express functional Toll-like receptor 2 (TLR2) and TLR4 on the surface (37–39). These receptors play crucial roles in host recognition and the NF-κB signaling pathway and are used by many lactic acid bacteria to induce anti-inflammatory activity. TLRs and their adaptors are involved in lung defense mechanisms during *K. pneumoniae* infection (16, 17, 40, 41). In view of these elements, we can hypothesize that *L. plantarum* CIRM653 interacts with the TLR pathway to block NF-κB activation, thereby limiting the lung innate immune response during *K. pneumoniae* infection.

All these findings explain the innate immune response but provide no information about the adaptive pathway involved in the response to beneficial bacteria. To assess this question, we showed that oral administration of *L. plantarum* CIRM653 decreased *il2* (T cell growth factor) *T-bet* (Th1) and *ROR-*γ*t* (Th17) expression and increased *foxp3* (Treg) and *il10* expression in the animals' lungs at 72 h postinfection. *In vitro*, *L. plantarum* CIRM653 induced IL-10 production and decreased IL-23 synthesis by *K.*

pneumoniae-infected DCs, without any significant effect on IL-12p70 production. In experiments using *K. pneumoniae*-infected PBMCs treated with *L. plantarum* CIRM653, the biological relevance of elevated IL-10 levels was evidenced by decreases in IFN-γ and IL-17A levels and in T cell proliferation. Several studies have shown that IL-10 acts directly on $CD4^+$ T cells by inhibiting proliferation and the production of IL-2, IFN-γ, and TNF-α (42–44). We therefore hypothesized that the immunomodulatory effects of *L. plantarum* CIRM653 via the release of the anti-inflammatory cytokine IL-10 lead to the antiproliferative and inhibitory effects on T cells observed during *K. pneumoniae* infection. In addition, our results showed that *L. plantarum* CIRM653 induced Treg cells in the mediastinal lymph nodes of infected mice. Two main mechanisms have been suggested to explain the effect of beneficial bacteria on T cell proliferation. *Lactobacillus* can suppress proliferation either by directly interacting with the TLR signaling pathway in T cells (45, 46) or by inducing Treg differentiation and Treg cytokine (IL-10 and transforming growth factor β [TGF-β]) production (47, 48). Collectively, these findings suggest that *L. plantarum* CIRM653 exerts its immunosuppressive effect through IL-10 produced by Treg cells.

On the basis of several studies, IL-10 has emerged as a key immunoregulator during respiratory infection (49–52). A recent study on *K. pneumoniae* showed that IL-10 is required for bacterial clearance, reduction of lung tissue damage, and host survival (53). Regarding other pulmonary pathogens, Salva et al. reported that oral administration of *Lactobacillus rhamnosus* CRL1505 confers resistance to pulmonary infection by *Streptococcus pneumoniae* in a mouse model via increased production of IFN-γ, IL-6, IL-4, and IL-10 (33). Using a similar model, experiments also evidenced the protective effect of oral administration of *Lactobacillus casei* CRL431 on *Pseudomonas aeruginosa* infection and the beneficial effect of *Bifidobacterium longum* 5^{1A} via IL-10 induction during *K. pneumoniae* infection (17, 54).

Our observations and those in other reports, taken together, show that IL-10 production by *L. plantarum* CIRM653 following *K. pneumoniae* infection alleviates both the innate and adaptive pulmonary immune response, thereby lending weight to the concept of the gut-lung axis and strengthening the hypothesis of distal immunomodulation by beneficial bacteria. However, the decrease in inflammatory responses in the context of infectious diseases could be deleterious. Uncontrolled production of Th1 and Th17 cells can lead to dysregulated production of proinflammatory cytokines and chronic inflammation, possibly contributing to tissue damage and pathogen dissemination (55, 56). The oral administration of beneficial bacteria in the treatment of lung diseases is a promising strategy, but its benefit/risk ratio should be carefully assessed before any widespread implementation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Lactobacillus plantarum* CIRM653 was cultured in De Man-Rogosa-Sharpe (MRS) medium (Becton, Dickinson, Franklin Lakes, NJ, USA) under anaerobic conditions (Gas-Pak system; Bio-Rad, Hercules, CA, USA) at 37°C overnight. *Klebsiella pneumoniae* LM21 was grown overnight aerobically in brain heart infusion medium (Becton, Dickinson) at 37°C. Bacterial cells were harvested by centrifugation (11,000 $\times$ *g* for 10 min), and the pellet was resuspended in the appropriate cell culture medium. The optical density (OD) was measured at 620 nm to adjust the final concentration of the bacterial suspension, and the exact number of CFU was determined by plating serial dilutions of the inoculum onto MRS or Drigalski plates (Becton, Dickinson).

For experiments with peripheral blood mononuclear cells (PBMCs) or DCs, the bacterial cells were inactivated by exposure to UV for 1 h. Successful inactivation of bacteria was assessed by plating the final suspension on agar plates.

Murine pneumonia model. Animal experiments were performed with 6- to 8-week-old C57BL/6J mice (Charles River, Wilmington, MA, USA) after approval by the ethics committee of Auvergne (Comité d'Ethique en Matière d'Expérimentation Animale Auvergne, C2EA-02), in accordance with the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (directive 86/609/EEC) (APAFIS number 12731-2017102014013220 v4). A total of 0.1 ml of $1.75 \times 10^8 \pm 0.25 \times 10^8$ CFU of *L. plantarum* CIRM653 or phosphate-buffered saline (PBS) was administered intragastrically daily for 7 days. The study groups were as follows: the control group (PBS), the *L. plantarum* CIRM653 strain (*L. plantarum*) group, the *K. pneumoniae* LM21 strain (*K. pneumoniae*) group, and the *L. plantarum* CIRM653 strain and *K. pneumoniae* LM21 strain (*K. pneumoniae* and *L. plantarum*) group. On day 7, the mice were anesthetized with 0.2 ml of a solution

containing xylazine (0.2 mg/ml) and ketamine (50 mg/ml) and then infected by intranasal inoculation of 25 μl of a suspension containing $1.62 \times 10^6 \pm 0.78 \times 10^6$ CFU of *K. pneumoniae*.

The animals were euthanized 24 h or 72 h after infection. Bronchoalveolar lavage (BAL) was performed by inserting and collecting 1 ml of PBS through a 1.7-mm catheter in a 1-ml syringe. The number of total leukocytes in the BAL fluid was determined after centrifugation with a Neubauer counting chamber. Differential counts were obtained from cytospin preparations and stained with May-Grünwald stain (Sigma-Aldrich, St. Louis, MO, USA). Tissue homogenates were placed on ice and used for determining the number of CFU, gene expression levels, and cytokine concentrations in lungs.

Isolation of lung immune cells. Lungs or mediastinal lymph nodes (MLNs) were isolated, cut into small pieces, and incubated with 1.5 mg/ml collagenase, 0.1 mg/ml DNase, and 0.75 mg/ml hyaluronidase (all from Sigma-Aldrich) for 60 min at 37°C in a shaker. The tissues were mashed, passed through a 70-μm cell strainer, and washed in complete RPMI 1640–3% fetal calf serum (FCS) (BioWest, Abcys, Paris, France). Cells were centrifuged at 1,200 rpm for 5 min, washed again, passed through a 40-μm cell strainer, and finally suspended in complete RPMI 1640–20% FCS medium (Difco, Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of monocyte-derived dendritic cells. Human PBMCs were isolated from buffy coats of healthy donors by Ficoll-Histopaque (Sigma-Aldrich) density gradient centrifugation as previously described (57). Briefly, monocytes were purified from PBMCs by negative selection with the EasySep human monocyte enrichment kit as recommended by the manufacturer (StemCell Technologies, Vancouver, Canada). Immature monocyte-derived dendritic cells (MoDCs) were maintained in 10% FCS–RPMI 1640 supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (800 U/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) and IL-4 (400 U/ml; Miltenyi Biotec) for 5 days alone or in the presence of UV-inactivated bacteria at an MOI of 1 to 100 for 48 h.

Informed consent was obtained by the local French blood agency (Etablissement Français du Sang [EFS], Saint-Etienne, France) from all volunteers involved in the study.

Lymphocyte proliferation assay. Lymphocyte proliferation was measured by a [^{3}H]thymidine incorporation assay as previously described (58). Briefly, PBMCs were seeded in 96-well plates (1×10^6 cells per ml) and stimulated with 2 μg/ml of PHA (Sigma-Aldrich). UV-inactivated bacteria (MOI of 1 to 100) were added to PHA-treated wells for 3 days. Following treatment, [^{3}H]thymidine (Perkin-Elmer, Waltham, MA, USA) was added at a concentration of 1 μCi/well, and plates were incubated for an additional 4 h. The cells were then collected under a vacuum onto Whatman filter paper, and the incorporation of tritiated thymidine was measured with a β counter (Tri-Carb 2300TR; Canberra-Packard, Schwadorf, Austria). Proliferation results were expressed as mean counts per minute from triplicate measurements.

Cells without PHA or bacteria were used as negative controls, and cells with PHA and without bacteria were used as positive ones.

Cultures of epithelial cells and infections. Human lung carcinoma cells (A549; ATCC CCL185) were grown to confluence in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FCS and 1% penicillin and streptomycin. The human colonic epithelial cell line (Caco-2; ATCC HTB-37) was maintained in Dulbecco's modified Eagle's medium (DMEM), 20% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin and streptomycin. For bacterial infection, cells were seeded in 24- or 6-well tissue culture plates (Thermo Fisher Scientific) and incubated with *K. pneumoniae* and/or *L. plantarum* CIRM653 at a multiplicity of infection of 1 to 100 in complete medium without antibiotics. Following infection, cells were washed, and (i) RNA or proteins were extracted from fixed cells; (ii) adhesion was monitored by the addition of a 0.1% Triton X-100 solution, and the number of viable bacteria was determined by plating serial dilutions of the suspensions onto MRS agar plates; or (iii) fresh complete medium containing antibiotics was added for 18 h to determine cytokine secretion in the supernatants.

RT-quantitative PCR. For analysis of gene expression, total RNA was prepared from whole lungs or from cell culture by TRIzol extraction according to the manufacturer's recommendations (Life Technologies-Invitrogen, Monza, Italy). The quantity and quality of RNA were assessed by the ratio of the absorbance at 260/280 nm with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). cDNA was obtained with a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed to amplify mouse IL-2 (forward primer 5′-CCTGAGCAGGATGGAG AATTACA-3′ and reverse primer 5′-TCCAGAACATGCCGCAGAG-3′), mouse ROR-γt (forward primer 5′-TC TCTGCAAGACTCATCGACAAG-3′ and reverse primer 5′-GCTAAGCAGTTGGTGGTGCA-3′), mouse T-bet (forward primer 5′-GCCAGGGAACCGCTTATATG-3′ and reverse primer 5′-GACGATCATCTGGGTCACATTG T-3′), mouse FOXP3 (forward primer 5′-GCCAACGCCCCAACAAG-3′ and reverse primer 5′-CCCCGCCCAC CTTTTCT-3′), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5′-AACTTTG GCATTGTGGAAGG-3′ and reverse primer 5′-ACACATTGGGGGTAGGAACA-3′), human IL-8 (forward primer 5′-TAGCAAAATTGAGGCCAAGG-3′ and reverse primer 5′-AAACCAAGGCACAGTGGAAC-3′), human IL-6 (forward primer 5′-CCAGCTATGAACTCCTTCTC-3′ and reverse primer 5′-GCTTGTTCCTCACATCTCTC-3′), and human GAPDH (forward primer 5′-GGTGAAGGTCGGAGTCAACG-3′ and reverse primer 5′-CCATGTA GTTGAGGTCAATGAAG-3′). The PCRs were performed in 96-well plates in a total volume of 20 μl and with 20 ng of cDNA. The program was as follows: two initial steps at 50°C for 2 min and 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. Results were calculated by the comparative cycle threshold method and are expressed as relative mRNA expression compared with uninfected cells.

Flow cytometry. CD45$^+$ cells were positively selected from isolated lung or MLN cells using anti-CD45 microbeads (magnetically activated cell sorting [MACS]; Miltenyi Biotec), according to the manufacturer's instructions, and were stained with the following antibodies (all from MACS; Miltenyi Biotec) for 30 min at 4°C: CD11c-Alexa Fluor 700 (AF700), F4/F80-Brilliant Violet 510 (BV510), and major histocompatibility complex class II (MHCII)-phycoerythrin (PE) to analyze macrophages; lymphocyte

antigen 6 complex locus G6D (Ly6G)-peridinin chlorophyll protein (PerCP) Vio700 to analyze neutrophils; CD4-AF700 to analyze $CD4^+$ T cells; and Foxp3-PE to analyze Treg cells.

To assess the maturation status of the dendritic cells, the cells were stained with specific CD86 and DC-SIGN monoclonal antibodies or their isotype-matched controls (BD Biosciences, Le Pont de Claix, France) for 30 min at 4°C.

Cells were acquired on an LSR II instrument (Becton, Dickinson) and analyzed with FACSDiva software.

ELISA. The human cytokines IL-6, IL-8, IL-10, IFN-γ, IL-4, IL-17A, and IL-12p70 and the mouse cytokines KC, IL-6, TNF-α, IFN-γ, IL-10, and IL-23 were assayed in culture supernatants, BAL fluid, or lung homogenates for mouse experiments with enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, USA, and R&D Systems, Minneapolis, MN, USA, for humans and mice, respectively) according to the manufacturers' instructions.

Protein extraction and Western blotting. Cells were lysed with the NE-PER nuclear protein extraction kit (Thermo Fisher Scientific) from cell culture or with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) from whole lungs containing both a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Western blotting was performed with 20 μg of protein per lane. Membranes were probed with a rabbit anti-p65 NF-κB polyclonal antibody (1:1,000; BioLegend), a rabbit anti-IκBα polyclonal antibody (1:1,000; BioLegend), a rabbit anti-p65 NF-κB phosphorylated polyclonal antibody (1:1,000; BioLegend), or a β-actin polyclonal antibody (1:5,000; BioLegend) overnight at 4°C. After washes, the membranes were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase-conjugated IgG (Sigma-Aldrich). Signals were visualized with the Clarity Western ECL substrate (Bio-Rad). Quantification was performed with Image Lab 2.0 software (Bio-Rad).

Statistical analysis. One-way analysis of variance (ANOVA) (Kruskal-Wallis test with Dunn's multiple-comparison test) was performed with GraphPad Prism 6 software. A P value of <0.05 was considered statistically significant. Error bars depict means $\pm$ standard errors of the means (SEM).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00570-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare that no conflict of interest exists.

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